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Report on the Verification of the Performance of MON 87705 and MON 89788 Event-specific PCR-based Methods applied to DNA Extracted from GM Stack Soybean MON 87705 x MON 89788

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This document replaces (same title) with ISBN number 978-92-79-36883-7 and PUBSY request JRC89449. The correction made in the new document is an update of the list of authors.

JRC90804

EUR 26589 EN

ISBN 978-92-79-36883-7 (PDF)

ISSN 1831-9424 (online)

doi:10.2788/47016

Luxembourg: Publications Office of the European Union, 2014

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Printed in Italy



EUROPEAN COMMISSION JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection

Molecular Biology and Genomics Unit



Report on the Verification of the Performance of MON 87705 and MON 89788 Event-specific PCR-based Methods applied to DNA Extracted from GM Stack Soybean MON 87705 x MON 89788

14 March 2014

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company to request the authorisation of genetically modified (GM stack) soybean MON 87705 x MON 89788 (with improved fatty acid profile and glyphosate-tolerance) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 on GM Food and Feed. The unique identifier assigned to the GM stack MON 87705 x MON 89788 soybean is MON-877 \emptyset 5-6 × MON-89788-1.

The GM stack MON 87705 x MON 89788 soybean has been obtained by conventional crossing of two genetically modified single line soybean events: MON 87705 and MON 89788 without any new genetic modification.

The EU-RL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single line soybean events MON 87705 and MON 89788 and has published the corresponding reports (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min Perf Requirements Analytical methods.pdf) the EU-RL GMFF therefore has carried out only an *in-house* verification of the performance of each validated method when applied to DNA extracted from the GM stack MON 87705 x MON 89788 soybean.

The hereby reported *in-house* verification study led to the conclusion that the individual methods meet the ENGL requirements also when applied to DNA extracted from the GM stack MON 87705 x MON 89788 soybean.

This report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

Quality assurance

The EU-RL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

The EU-RL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by CERMET.

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1. Introduction

The EU legislative system ^(1, 2) for genetically modified food and feed provides that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (GM stack).

Consequently, the application for authorisation of a GM stack shall be accompanied, among others, by an event-specific method for detection, identification and quantification of each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EU-RL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EU-RL GMFF report in the overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined bv the ENGL (http://gmocrl.jrc.ec.europa.eu/doc/Min Perf Requirements Analytical methods.pdf) the EU-RL GMFF carries out an *in-house* verification of the performance of each event-specific methods if this method has previously been validated by the EU-RL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack MON 87705 x MON 89788 soybean.

Upon reception of methods, samples and related data (step 1), the EU-RL GMFF carried out the assessment of the documentation (step 2) and the in-house verification of the methods (step 3) according to the requirements of Regulation (EC) No 641/2004 (Annex I).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Monsanto Company submitted the detection methods and the corresponding control samples of the GM stack MON 87705 x MON 89788 soybean.

The dossier was found to be complete and thus was moved to step 2.

3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL ⁽³⁾ and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated on the basis of the data provided by the applicant for the two methods on the stack

DNA. Means are the average of fifteen replicates obtained through one run on ABI7500. Percentages are expressed as GM DNA / total DNA x 100.

Table 1. Estimates of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) provided by the applicant for the MON 87705 and MON 89788 methods applied to GM stack MON 87705 x MON 89788 soybean.

MON 87705							
Unknown sample GM%	Expected value (GMO %)						
	0.085	1.0	10				
Mean	0.071	0.94	8.8				
RSD _r (%)	14	6.8	4.3				
Bias (%)	-16	-6.3	-12				
мо	MON 89788						
	Expecte	ed value (G	MO %)				
Unknown sample GM%	0.085	1.0	10				
Mean	0.076	0.99	9.4				
RSD _r (%)	18	7.1	4.8				
Bias (%)	-10	-1.3	-5.9				

The EU-RL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL acceptance criteria ⁽³⁾.

No further requests of complementary information were addressed to the applicant; therefore the dossier was moved to step 3.

4. Step 3 (EU-RL GMFF experimental testing)

In step 3 the EU-RL GMFF implemented the two methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack MON 87705 x MON 89788 soybean.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenized seeds of GM stack MON 87705 x MON 89788 soybean;
- genomic DNA extracted from homogenized seeds of non-GM soybean.

The EU-RL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean with genomic DNA extracted from non-GM soybean in a constant amount of total soybean DNA. The same concentrations

as used in the validation of the methods for the parent single lines were achieved. Table 2 shows the five GM concentrations used in the verification of the MON 87705 and MON 89788 methods when applying them to genomic DNA extracted from the GM stack MON 87705 x MON89788 soybean. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage of MON 87705 and MON 89788 in MON 87705 \boldsymbol{x} MON 89788 in the verification samples.

MON 87705 GM % (GM DNA / total DNA x 100)	MON 89788 GM % (GM DNA / total DNA x 100)
0.1	0.1
0.5	0.4
0.9	0.9
5.0	4.0
8.0	8.0

The protocols (reagents, concentrations, primers/probes sequences) described by the applicant were implemented precisely in the EU-RL GMFF laboratory. The *in-house* verification followed exactly the protocols already published as validated methods for the individual MON 87705 and MON 89788 single events (available at <u>http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx</u>).

4.2 DNA extraction

A method for DNA extraction from soybean seeds was previously evaluated by the EU-RL GMFF with regards to its performance characteristics and was considered valid i.e. fit for the purpose of providing soybean DNA of appropriate quality and amount for being used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/MON89788_Soya_DNAExtrSampl_report.pdf.

Consequently, the EU-RL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs for each method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system lectin (*Le1*). Five GM levels were examined per run, for each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 87705 and MON 89788), the quantification of the five GM levels was calculated as an average of sixteen replicates per GM level (8 run x 2 replicated levels per run). An Excel spreadsheet was used for determination of GM%.

4.4 PCR methods

During the verification study, the EU-RL GMFF carried out tests on DNA extracted from GM stack MON 87705 x MON 89788 soybean, using the methods previously validated for the respective single line GM soybean events MON 87705 and MON 89788, respectively.

For the detection of GM soybean events MON 87705 and MON 89788, DNA fragments of 86bp and 139-bp respectively are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: FAM (6-carboxyfluorescein) is used as reporter dye at its 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3'-end.

For quantification of GM soybean events MON 87705 and MON 89788, a taxon-specific reference system amplifies a 74-bp fragment of lectin (*Le1*) soybean endogenous gene (GenBank K00821), using two *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM and TAMRA.

For relative quantification of GM soybean events MON 87705 and MON 89788 DNA, respectively in a separate test sample, standard curves are generated both for the MON 87705 and MON 89788 and the *Le1* specific system by plotting Ct values of the calibration standards against the logarithm of the target DNA copy numbers and by fitting a linear regression into these data. The normalised Ct values of the unknown samples are measured and, by means of the regression formula, the relative amount of MON 87705 and MON 89788 DNA, respectively, is estimated.

For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at <u>http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx</u>.

4.5 Deviations from the validated methods

No deviations from the original validated methods were introduced.

4.6 Results

Tables 3 and 4 present the values of the slopes of the different standard curves generated by the EU-RL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the R² (expressing the linearity of the regression) reported for all PCR systems in the eight runs.

		MON 8770	5		Le1	
Run	Slope	PCR Efficiency (%)	Linearity (R ²)	Slope	PCR Efficiency (%)	Linearity (R ²)
1	-3.25	103	1.00	-3.35	99	1.00
2	-3.22	105	1.00	-3.39	97	1.00
3	-3.26	103	1.00	-3.34	99	1.00
4	-3.34	99	1.00	-3.33	100	1.00
5	-3.37	98	0.99	-3.34	99	1.00
6	-3.28	102	1.00	-3.35	99	1.00
7	-3.35	99	1.00	-3.36	98	1.00
8	-3.38	97	1.00	-3.35	99	1.00
Mean	-3.30	101	1.00	-3.35	99	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 87705 method on GM stack MON 87705 x MON 89788 soybean.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 89788 method on GM stack MON 87705 x MON 89788.

	MON 89788			Le1			
Run	Slope	PCR Efficiency (%)	Linearity (R ²)	Slope	PCR Efficiency (%)	Linearity (R ²)	
1	-3.43	96	0.99	-3.38	98	1.00	
2	-3.37	98	1.00	-3.36	98	1.00	
3	-3.36	99	1.00	-3.30	101	1.00	
4	-3.57	91	0.99	-3.26	102	1.00	
5	-3.50	93	1.00	-3.50	93	1.00	
6	-3.43	96	1.00	-3.35	99	1.00	
7	-3.50	93	0.99	-3.29	101	1.00	
8	-3.41	97	1.00	-3.43	96	1.00	
Mean	-3.45	95	0.99	-3.36	99	1.00	

The mean PCR efficiencies of the calibration curves for each of the two event-specific methods were above 90% (101% and 95% for MON 87705 and MON 89788 respectively). The mean PCR efficiency of the *Le1* reference gene method was 99%. The linearity of the methods (R²) was 1.00 for MON 87705 and 0.99 for MON 89788 respectively, while was 1.00 for the *Le1* method in both cases. The data generated by the EU-RL GMFF and presented in Tables 3 and 4 are in line with the data presented by the applicant and confirm the appropriate performance characteristics of the two methods when tested on DNA extracted from the GM stack MON 87705 x MON 89788 soybean in terms of PCR efficiency and linearity.

The EU-RL GMFF also assessed the values of trueness and precision (expressed as RSDr %, relative repeatability standard deviation), of the two methods applied to samples of genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean, see table 5 and 6.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87705 method applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean.

MON 87705						
Unknown	Expected value (GMO%)					
sample GM%	0.1	0.5	0.9	5.0	8.0	
Mean	0.10	0.45	0.98	5.0	8.6	
SD	0.01	0.05	0.17	0.59	0.75	
RSD _r (%)	11	11	17	12	8.7	
Bias (%)	-2.3	-9.7	9.0	0.77	7.6	

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89788 method applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean.

MON 89788						
Unknown		Expected value (GMO%)				
sample GM%	0.1	0.4	0.9	4.0	8.0	
Mean	0.10	0.37	0.91	3.9	7.9	
SD	0.01	0.06	0.11	0.60	0.79	
RSD _r (%)	8.8	17	12	16	10.0	
Bias (%)	-3.0	-7.6	1.1	-3.7	-1.1	

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method should be \pm 25% across the entire dynamic range. As shown in Tables 5 and 6, the values range from -9.7% to 9.0% for MON 87705 and from -7.6% to 1.1% for MON 89788. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean.

Tables 5 and 6 also show the relative repeatability standard deviation (RSD_r) for each GM level. According to the ENGL acceptance criteria and method performance requirements, RSD_r values should be below 25%. As the values range between 8.7% and 17% for MON 87705 and between 8.8% and 17% for MON 89788, the two methods satisfy this requirement throughout their respective dynamic ranges also when applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean.

5. Comparison of method performance when applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean and to DNA extracted from the single-line GM events

An indicative comparison of the performance (bias, RSDr %) of the two methods applied to GM stack MON 87705 x MON 89788 soybean and to the single-line events is shown in Tables 7 and 8. The performance of the methods on the single lines was previously validated through international collaborative trials (<u>http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx</u>).

Note: the comparison of data generated in different testing conditions and different times is intended to be only of qualitative nature; differences in the figures reported are not necessarily statistically significant.

Table 7. Qualitative comparison of the performance of the MON 87705 detection method applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean and to genomic DNA extracted from the single line event MON 87705.

87705 q	ess and repeatabil uantification on G 705 x MON 89788	M-Stack MON		and repeatability ion on single ever	
GM%	Bias (%)	RSD _r (%)	GM%	RSD _r (%)	
0.1	-2.3	11	0.1	-5.0	20
0.5	-9.7	11	0.5	0.71	15
0.9	9.0	17	0.9	-3.4	15
5.0	0.77	12	5.0	-4.5	15
8.0	7.6	8.7	8.0	-3.4	13

*Data taken from original method validation (<u>http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm</u>)

Table 8. Qualitative comparison of the performance of the MON 89788 detection method applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean and to genomic DNA extracted from the single line event MON 89788.

	ess and repeatabi uantification on M MON 89788	-		and repeatability o ion on single even	
GM% Bias (%) RSD _r (%)			GM%	Bias (%)	RSD _r (%)
0.1	-3.0	8.8	0.1	-14	16
0.4	-7.6	17	0.4	-5.0	22
0.9	1.1	12	0.9	-0.9	15
4.0	-3.7	16	4.0	11.0	13
8.0	-1.1	10.0	8.0	2.8	12

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

6.Conclusions

The performance of the two event-specific methods for the detection and quantification of soybean events MON 87705 and MON 89788, when applied to genomic DNA extracted from GM stack MON 87705 x MON 89788 soybean, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL.

In conclusion, the verification study carried out by the EU-RL GMFF confirmed that the two methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack MON 87705 x MON 89788 soybean.

Therefore these methods, originally developed and validated to detect and quantify the events in the single event parental GMOs, can be equally applied for the detection and quantification of the respective events combined in GM stack MON 87705 x MON 89788 soybean.

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European Commission EUR 26589 EN – Joint Research Centre – Institute for Health and Consumer Protection

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Author(s): Sara Jacchia, Maria Grazia Sacco, Cristian Savini, Marco Mazzara, Joachim Kreysa

Luxembourg: Publications Office of the European Union

2014 – 16 pp. – 21.0 x 29.7 cm

EUR - Scientific and Technical Research series -ISSN 1831-9424 (online)

ISBN 978-92-79-36883-7 (PDF)

doi:10.2788/47016

Abstract

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